raise from delocalization, among several BChl molecules, of the charge associated with the oxidized BChl. A delocalization of this sort can be used as the conduction system for separation of charge in the primary quantum conversion act.

We would like to express our appreciation for the technical assistance given by Miss Ann Maksim and invaluable discussion with Dr. M. P. Klein.

* The work described in this paper was sponsored in part by the U.S. Atomic Energy Commission.

† National Science Foundation fellow, 1962–1964.

[1] Calvin, M., and G. M. Androes, *Science*, **138**, 867 (1962).

[2] Commoner, B., in *Light and Life*, ed. W. D. McElroy and B. Glass (Baltimore: Johns Hopkins Press, 1961), p. 356.

[3] Clayton, R. K., *Photochem. and Photobiol.*, **1**, 201 (1962).

[4] Duysens, L. N. M., in *The Photochemical Apparatus—Its Structure and Function*, Brookhaven Symposia in Biology, No. 11 (1958), p. 325.

[5] Loach, P. A., G. M. Androes, A. F. Maksim, and M. Calvin, *Photochem. and Photobiol.*, **2**, 443 (1963).

[6] Androes, G. M., *Adv. Bot. Research*, **1**, 326 (1963).

[7] Kuntz, I. D., Jr., P. A. Loach, and M. Calvin, *Biophys. J.*, in press.

[8] Bell, W. E., A. L. Bloom, and J. Lynch, *Rev. Sci. Inst.*, **32**, 688 (1961).

[9] Klein, M. P., and G. W. Barton, *Rev. Sci. Inst.*, **34**, 754 (1963).

[10] Cohen-Bazire, W., R. Sistrom, and R. Y. Stanier, *J. Cell. Comp. Physiol.*, **49**, 29 (1957).

THE DISTRIBUTION OF BUOYANT DENSITY OF HUMAN ERYTHROCYTES IN BOVINE ALBUMIN SOLUTIONS

BY ROBERT C. LEIF AND JEROME VINOGRAD

GATES AND CRELLIN LABORATORIES OF CHEMISTRY* AND THE NORMAN W. CHURCH LABORATORY OF CHEMICAL BIOLOGY, CALIFORNIA INSTITUTE OF TECHNOLOGY

Communicated by Norman Davidson, January 2, 1964

Several reports have appeared in the literature of the variation of "density" among different types of dispersed cells and of physical separations based upon this property.[1, 2] Two general separation procedures have been used. *Packed cell methods:* cells are centrifuged from a suspension to form a viscous mass of packed cells, which is separated layerwise. *Neutral density separations:* cells are centrifuged in a dense liquid medium and are segregated into two fractions, one denser and one lighter than the suspending medium. With such methods the order of density of certain types of blood cells has been established.[3–5] It has also been shown that erythrocytes are heterogeneous in density and that young erythrocytes are less dense than old.[6–9]

The above procedures are not suitable for the quantitative investigation of the density distribution in cell populations. The packed cell mass is difficult to fractionate. The neutral density procedure yields only two fractions in each experiment. These restrictions may be overcome by centrifuging the cells in a buoyant density gradient.[2, 10, 11]

The present communication describes a method for the quantitative fractionation of erythrocytes in a linear density gradient of bovine serum albumin. It is shown

that cells segregate in the density gradient on the basis of buoyant density, a quantity defined as the density of the fluid in which the cell is neutrally buoyant. Layerwise fractionation of the tube contents provided material for the analysis of the density distribution and for studies of osmotic behavior, cell volume distribution, red cell aging, and cytological variation.

Materials and Methods.—Preparation of the gradients and the sampling of the tubes: Linear density gradients, *ca.* 0.006 gm cm^{-4}, were prepared from bovine serum albumin (BSA) solutions with a density gradient machine based on a Technicon peristaltic pump (*P*, Fig. 1). The same

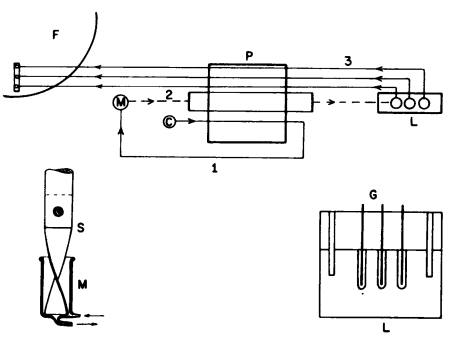

FIG. 1.—Equipment for formation and fractionation of BSA gradients. The plastic tubing is 0.030" ID except for the dashed lines, which are 0.040." The pumping tubes were obtained from the Technicon Company. The stirrer blade S was fashioned from Teflon sheet 1.5 × 0.15 × 6.5 cm by twisting 180° over 5 cm. The stirrer is mounted with a Teflon pin on a grooved Lucite rod, and rotated 150 rpm. The dimensions of the glass mixing chamber are 1.7 cm ID × 3.0 cm. The glass capillary tubes, G, 1.14 mm OD, 1.0 mm ID × 12 cm, were obtained from the Kensington Scientific Corporation, Berkeley, California (100 λ Microcaps). The speed of the Technicon pump, Model No. 1, was changed by a factor of 9/16 by exchanging sprockets 37V and 38V.

pump was used as a tube sampler for the recovery of equal size fractions. These operations, the preparation of BSA solutions, the preparation of the cell samples, and the centrifugation were performed at 4°. Tygon tube 1, filled and flushed with concentrated BSA, was connected to the inlet of the dry 5-ml mixing chamber, *M*. A 2.35-ml volume of dilute BSA was pipetted into the mixer. Concentrated BSA (*C*) was pumped into the mixer and the mixed solution into the centrifuge tube mounted in a Lucite block (*L*). The arrangement of Tygon tubes 1 and 2 is such that the output from the mixer is twice the input, the condition for the formation of a linear density gradient.[12, 13] The BSA solutions were delivered to the bottom of the centrifuge tube through a glass capillary (*G*). Filling required 11 min and was stopped before efflux of the first bubble.

After centrifuging, the tubes were again mounted in the Lucite block. Fourteen equal fractions and the remainder were pumped out through Tygon tube 3, into 10 × 75-mm test tubes in a fraction collector, actuated at 1.4 min intervals. Brushing of the Tygon delivery tubing against the test tube rim fractionated the last drop. The volume of each fraction was 0.314 ± 0.005 ml.

Application of the cells: Three drops of blood obtained by finger punch were dripped into 0.4 ml. of Alsever's[14] solution. Within 20 min, 0.30 ml of this suspension was layered onto the density gradient column. In the aging experiments the cells, suspended in Alsever's solution, were packed in a clinical centrifuge, freed of most of the supernatant fluid by decantation, spun again, and the remaining fluid was absorbed into a wad of tissue. The pellet was first dispersed and then blended into 3 ml of the dense BSA solution with a Vortex mixer. Introducing the cells as a top layer gives rise to a greater contamination of the light layers by cells which are driven to the cylindrical walls by the radial field. Rouleaux formation was absent in suspensions of cells in BSA.

Centrifugation: The SW 39 tubes were centrifuged in a Model L Spinco ultracentrifuge at 20,000 rpm for 60 min. To prevent swirling, the heavier SW 25 rotor fitted with special nylon adapters was used and was accelerated and decelerated as recommended by de Duve *et al.*[15] In the aging experiments the SW 39 tubes were spun in a Servall HS field-aligning rotor at 12,000 rpm for 60 min. The rotor was accelerated manually in 10 min and decelerated without braking.

Density measurements: Densities were measured in a 0.224-ml pycnometer made by removing the overflow bulb and drawing out both ends of a 0.3-ml micropipet. The pycnometer was completely filled by suction with a side tube and pinch clamp in the suction line. The volume of the pycnometer was determined with water with a precision of ± 5 × 10^{-5} ml. The standard

error in the determination of the density of the BSA solutions was ± 0.00045. Upon filling the pycnometer, gas bubbles formed in the BSA solutions. These rose to the top of the pycnometer and were drawn into the suction line after 5–20 min. All density data reported in this communication were corrected to 4° with expansion coefficients, 2.9 and 2.6 × 10^{-4} gm cm^{-3}/°C, obtained by dilatometry of 1.106 and 1.073 gm cm^{-3} BSA solutions, respectively.

Determination of the optical density profile: The cells were lysed by adding 2.5 ml of CO-saturated distilled water, inverting the tubes a few times, and stirring with a Vortex mixer for 30 seconds. The lysates were centrifuged at low speeds to remove debris and read at 518 mμ[16] in a Zeiss PMQ spectrophotometer. The results were corrected for the absorbance of the BSA in the fraction. In the aging experiment the fractions were first diluted with CO-saturated NKM[17] solution and spun in a clinical centrifuge. The supernatant was decanted, and the cells were lysed with 2.5 ml of 0.05 μg/ml saponin solution.

Preparation of the BSA solutions: Armour and Co., Chicago, bovine albumin powder, fraction V, was used throughout this work. One of the dry powders contained 2.65% water and 1.60% ash. The deionized concentrated BSA solution was prepared as follows: 108 gm of the powder were layered on 200 gm of water containing 50 gm of Amberlite MB-3 resin in a one-half gallon polyethylene refrigerator container provided with a magnetic stirring bar and a tight lid. The container was tapped lightly every few hours as needed and finally tilted to bring the BSA powder into contact with liquid. The solution decanted from the resin was centrifuged at 1,000 rpm in 250-ml centrifuge bottles, decanted into another centrifuge bottle which contained 25 gm of resin, intermittently agitated over a 2-hr period, and centrifuged in stainless steel tubes for 2 hr at 20,000 rpm in the Spinco 30 rotor. The top layers were removed with a Pasteur pipet, and the decanted solutions pooled in a tared vessel.

The following salts were added per 100 gm of deionized BSA solution: 0.2391 gm Na_2CO_3, 0.3030 gm NaCl, 0.1124 gm $MgCl_2 \cdot 6H_2O$, 0.0286 gm KCl. The above mixture neutralizes the BSA and provides the cation composition and the osmolarity of NKM as calculated for the 67% water present. The densities of the final solutions were 1.104 ± 0.001 gm cm^{-3}. The pH determined with pH paper was 6.8. The dilute solutions were made up by weight assuming additive volumes. All solutions were stored at −60° to prevent deterioration of BSA.

The BSA solutions for the tonicity experiments were prepared from deionized concentrated BSA ρ = 1.103 by addition of 0.106 ml of a salt solution (12.5 gm KCl and 49.13 gm $MgCl_2 \cdot 6H_2O$ per 100 ml) to 100 gm of BSA solution containing 0.2391 gm of Na_2CO_3. This stock solution contained the same cation proportions as NKM and had 40.4% of the osmolarity. A 25 × concentrated NKM solution was added to aliquots of the stock solution to obtain solutions ranging from 80 to 120% of the osmolarity of NKM. The concentrated solutions were diluted with NKM solutions of the corresponding osmolarity to form the dilute BSA solutions.

In the aging experiments the BSA contained a mixture of solutes approximating Eagle's saline[18] but at pH 6.8. The BSA-solute mixture was prepared by mixing 100 gm BSA solution with 0.5 ml of a slurry obtained by evaporating under dry N_2 13.33 ml of a solution containing in gm/l: 25.10 Na_2CO_3, 16.56 NaCl, 2.08 KCl, 0.74 $NaH_2PO_4 \cdot H_2O$, 5.23 dextrose and 2.92 streptomycin sulfate, and 5 × 10^5 units/l of penicillin SK. Finally 0.261 ml each of 54 gm/100 ml $CaCl_2$ and 45 gm/100 ml $MgCl_2 \cdot 6H_2O$ were added. The dilute BSA was prepared by adding a modified Eagle's saline solution. This solution was prepared by titrating Eagle's saline into a solution of the same composition except for the replacement of the $NaHCO_3$ by 1.39 gm/l NaCl and the above antibiotics (streptomycin sulfate 0.5 gm/l and 1 × 10^5 units penicillin) to a pH of 6.8.

All chemicals were reagent grade. The water was redistilled in glass. Alsever's sterile solution was supplied by Delco Chemical Company, Glendale, California.

Determination of Fe^{59}: The saponin lysates were precipitated with 0.5 ml 50% TCA. The precipitate was washed once by centrifugation with 5% TCA, dissolved in 0.5 ml 50 V% acetic and formic acids, and assayed on glass planchettes with a Nuclear-Chicago low-background counter.

The distribution of cell volumes: The volume distributions were determined with a Model B Coulter counter according to Brecher *et al.*[19] The fractions were diluted 10-fold in Eagle's saline, and aliquots diluted a further 200-fold just prior to the analyses performed in triplicate. The results were converted to cell volumes with a factor obtained from the hematocrit value.[19]

Cytological examination of the cells: The fractions, diluted 10-fold with Alsever's solution, were centrifuged; the packed cells were resuspended in 0.2 ml of homologous serum and again centri-

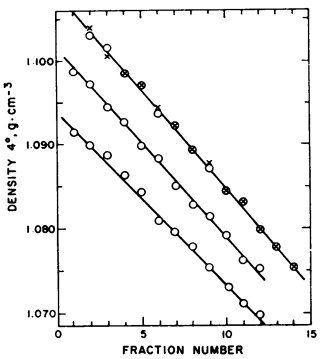

FIG. 2.—BSA density gradients. Densities were evaluated from refractometer readings with the relation, $\rho_4^4 = 1.0540 + 1.543\ (N_{25}^D - 1.3670)$. *Upper line:* solutions pumped directly from mixing chamber into fraction collector actuated at 0.70-min intervals. Two separate experiments, O, X. The ordinate values are displaced upward by 0.0040 gm cm^{-3}. *Middle line:* collected from centrifuge tube; not centrifuged. *Lower line:* centrifuged. Different BSA solutions were used in these experiments.

fuged. Slides prepared with Wright's stain or brilliant cresyl blue were examined with a Zeiss Ultraphot II microscope. The preparation and examination of the slides were performed in collaboration with Dr. D. Hammond and Mrs. L. Capers, Children's Hospital, Los Angeles.

Examination of the density gradient: The linearity of the density gradient was checked in several runs before and after centrifugation in absence of cells (Fig. 2). In buoyant density experiments the densities of the second, seventh, and twelfth fractions were determined pycnometrically. The density difference per fraction was obtained as the average of the result between the second and the seventh, and second and twelfth fractions. The density of each fraction was calculated from the density of the second fraction and the density difference per fraction. Greater weight was given to the second fraction because it is least affected by drainage errors. The average density difference between fractions 2 and 7 was 10% greater than between 7 and 12.

Density distributions of absorbance, radioactivity, and cell volume: The results of these experiments are given graphically as density distribution functions. The ordinate scale is the per cent of the total hemoglobin or radioactivity in the fraction divided by the density increment per fraction. This ordinate scale represents the normalized distribution function $F(\rho) = 1/g_0\ dg/d\rho$, where g is the amount of material in the fraction, and g_0 the total amount of material in the sample.

Means were calculated from distributions which had been truncated to eliminate the unreliable tails. Narrow distributions, with maxima, $F(\rho) > 10$, were truncated at $F(\rho) = 3$. Broader distributions were truncated at $F(\rho) = 2$. The cell volume distributions were not truncated for the calculation of mean values. The mean cell volumes were corrected for the experimentally determined dependence of the mean on the concentration of the cells in the sample.

Results and Discussion.—The buoyant density distribution curves for the erythrocytes from a single donor (R.C.L.) are shown in Figure 3*A*. These curves which give the results for three separate runs demonstrate the reproducibility of the method. The average mean of the distributions in 9 experiments performed over a period of one month was 1.0836 ± 0.00051 gm cm^{-3}. The spread of the means corresponds to about one fifth of a fraction. Veinous blood in Alsever's solution stored 24 hr at 4° gave substantially the same distributions.

That the observed buoyant density distributions in Figure 3 are real was shown

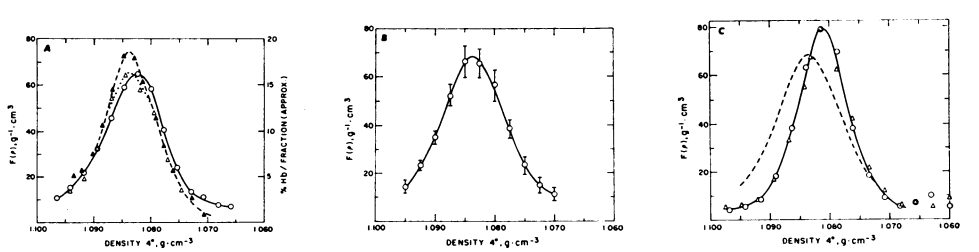

FIG. 3.—Buoyant density distributions of erythrocytes. (*A*) Erythrocytes from R.C.L. Three separate experiments. (*B*) Ordinates averaged at the indicated densities in nine experiments. The error bars indicate the standard deviation. (*C*) Comparison of (*B*) with results of two experiments from another donor, W. G.

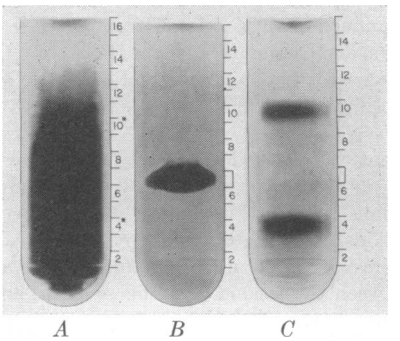

FIG. 4.—Rebanding experiment. Photographs of erythrocyte distributions: (*A*) Original distribution. (*B*) Mixture of 4* and 10* from (*A*). (*C*) After centrifugation of (*B*). Note that an extra layer of BSA is present in (*B*) and (*C*).

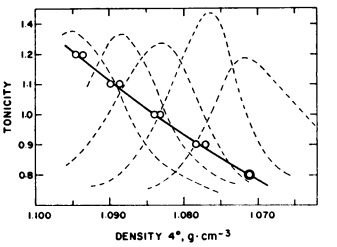

FIG. 5.—Effect of salt concentration on the buoyant density distribution of erythrocytes in BSA. The dashed lines give one set of observed distributions at relative salt concentrations listed from left to right, 1.2, 1.1, 1.0, 0.9, and 0.8. The experimental points represent the means of the distributions. The line was calculated with equation (1) and $\rho_{iso} = 1.0836$ gm cm^{-3}.

in "rebanding" experiments in which isolated density fractions were centrifuged a second time in a new density gradient. In such an experiment performed with a threefold normal concentration of cells, fractions 4 and 10 were collected in one test tube and mixed. The mixture was drawn into the dry pump lines and one fraction discharged at the isodensity position in a new BSA gradient. After centrifugation the new bands reappeared at the expected positions (Fig. 4).

The erythrocytes of four other normal individuals were examined. The means, 1.0809, 1.0804, 1.0808, and 1.0812 gm cm^{-3}, were significantly smaller than the mean, 1.0836, for the cells from R.C.L. The density difference corresponds to the observed difference in mean cell volume, 3.6 cu micra, if it is assumed that the volume change is the result of a change in water content (cf. legend, Fig. 7).

Erythrocytes of duck blood isolated by this fractionation procedure have been shown to incorporate radioactive RNA and protein precursors.[20]

Effect of tonicity on buoyant density: The well-known swelling and shrinking of erythrocytes in solutions of different tonicity (ratio of solute osmolarity to the osmolarity of plasma) may be expected to affect the buoyant density. A series of experiments at different salt levels was performed to examine this effect (Fig. 5). The entire distribution shifted with salt concentration. The relation between the shift as given by the mean of the distribution and the salt concentration is well represented by Ponder's equation 3.10[21] for the change in volume of cells which are assumed to be permeable to water, impermeable to at least one solute, and to obey van't Hoff's law. Ponder's equation expressed in terms of buoyant density is

$$\rho = \left[\left(\frac{1}{T} - 1\right)\rho_w + \left(\frac{V}{V_w}\right)_{iso}\rho_{iso}\right] \Big/ \left[\left(\frac{1}{T} - 1\right) + \left(\frac{V}{V_w}\right)_{iso}\right] \qquad (1)$$

where ρ is the buoyant density of a cell at tonicity T, and ρ_{iso} and $(V/V_w)_{iso}$ are the buoyant density, 1.0836 gm cm^{-3}, and the ratio, 1.43, of the total volume to the volume of water in a cell at unit tonicity.

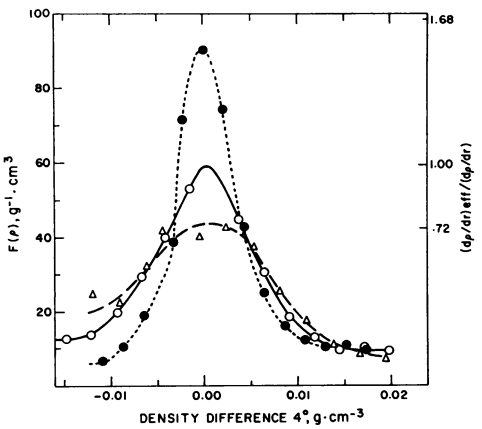

FIG. 6.—Effect of salt gradients on the buoyant density distribution of erythrocytes in a BSA density gradient. *Solid line:* distribution obtained with no salt gradient. *Dotted line:* distribution obtained with $(d\rho/dr)_{eff}/(d\rho/dr)$ = 1.68. Tonicities of the concentrated and dilute BSA solutions were 0.80 and 1.20, respectively. *Dashed line:* distribution with the above ratio = 0.72. Tonicities of the concentrated and dilute BSA solutions were 1.10 and 0.90, respectively.

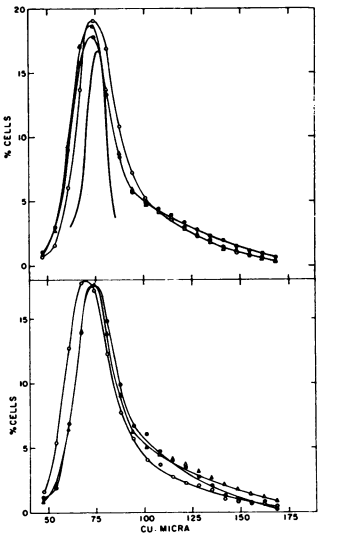

FIG. 7.—Volume distribution of erythrocytes by electronic size determinations. *Upper:* unfractionated cells. ● untreated cells; ○, △, cells suspended in concentrated BSA. Narrow band is volume distribution calculated from density distribution (Fig. 3*B*) with $V = (\rho - \rho_0/\rho_w - \rho)V_0 + V_0$, where ρ_0 and V_0 are the original density and volume, respectively, and ρ_w is the density of water. *Lower:* fractionated cells. ○, fraction 1; △ fraction 7; ●, fraction 12.

The effective density gradient in the BSA-erythrocyte-salt-water system: In this system the density gradient effective in resolving cells of different buoyant density is a composite of the real or physical density gradient set up by the BSA and a gradient in tonicity, dT/dr, associated with the distribution of any nonpermeable solutes. The effect of the BSA on the tonicity is negligible.[22] The tonicity experiments allow us to estimate the effect of unequal solute concentrations in the original dilute and concentrated BSA. The effective density gradient $(d\rho/dr)_{eff}$ has been shown[23] in buoyant density studies of dissolved macromolecules to be the sum of the physical density gradient, $d\rho/dr$, and a further density gradient which describes the response of the buoyant species to a change in solute activity, $(d\rho/dr)_{eff} = d\rho/dr - (da/dr)\ (d\rho/da)$, where a is the activity of the solute.[24] In these studies tonicity is assumed to be proportional to solute activity; the derivative $d\rho/dT$ may then be evaluated from Figure 5, or, after differentiation, from equation (1). At $T = 1$ the differential equation becomes $(d\rho/dT)_{iso} = (\rho - \rho_w)(v_w/v)_{iso}$. The derivative for the mean density of cells from R.C.L. is 0.059 gm cm^{-3}. Resolution is inversely related to the effective density gradient for $(d\rho/dr)_{eff}$

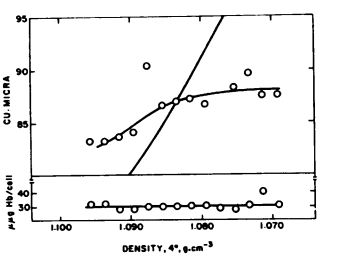

FIG. 8.—*Upper:* mean volumes of cells in the buoyant density fractions. The theoretical line was calculated from the equation in legend for Fig. 7. *Lower:* the amount of hemoglobin per cell.

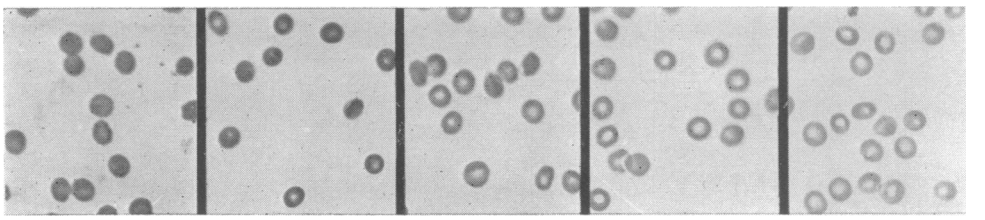

FIG. 9.—Microphotographs of erythrocytes stained with Wright's stain. From left to right, cells were obtained from the bottom pellet, and fractions 1, 8, and 12 in order of decreasing density, and from an unfractionated blood sample.

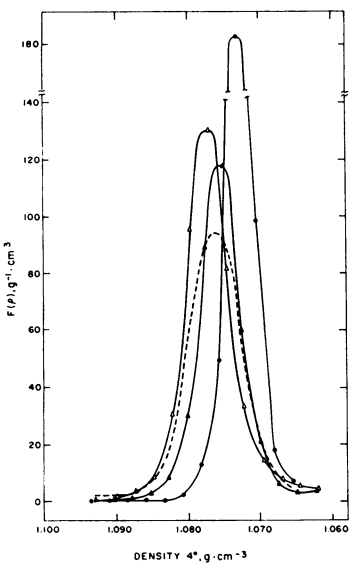

FIG. 10.—Buoyant density distributions of radioactive erythrocytes. ● Two days after injection of $Fe^{59}Cl_3$, ▲ 16 days, △ 35 days. The dashed line represents the erythrocyte distribution.

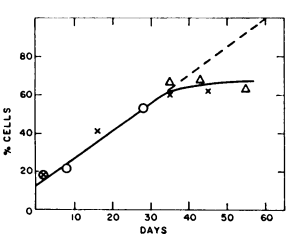

FIG. 11.—Progression of the mean of the radioactivity through the erythrocyte distribution. Results from three rabbits, ○, △, ×, are given. The ordinate represents the percentage of cells having lower density than mean density of the radioactive cells.

> 0. In Figure 6 the results of three experiments with approximately the same physical gradient but widely different effective density gradients are shown. The maxima in the distributions are seen to correspond as expected to $(d\rho/dr)_{eff}/(d\rho/dr)$.

The relationship between buoyant density and cell volume: It is apparent from previously published volume distributions[19] that the density distribution is too narrow to arise from a variable water content in cells of otherwise constant composition (Fig. 7). Volume distributions of a light, median, and dense fraction are as heterogeneous in volume as the unfractionated cell population. In Figure 8, the solid line is the mean cell volume calculated from the densities with the assumption that the change in cell volume is solely due to water. Experimentally, however, the mean cell volumes of the lighter fractions appear to remain constant, and the observed change of density in the denser fractions is only in part explainable by dehydration. The main factor responsible for the distribution of buoyant density is at present unknown. It cannot be the distribution of lipid because the over-all lipid content[28] of the erythrocytes is far too small. Whether changes in the structure of the water in the erythrocyte occur upon "aging" is unknown.

Microscope examination of the density fractions: The human erythrocytes in fractions 3–12 were not distinguishable from the unfractionated cells (Fig. 9). The cells in the densest fraction, 1, and in the resuspended bottom pellet were markedly less biconcave than the lighter cells. The reticulocytes formed a narrow distribution within the lightest quarter of the erythrocytes. The maximum was displaced by 2.5 fractions (Fig. 3*B*). The largest relative concentration of reticulocytes, 3.3 per cent, was displaced one fraction further. The white cells were found most frequently in the lighter

fractions. The possibility of selective loss of reticulocytes and white cells in the manipulations prior to the microscopic examination cannot be excluded.

The relationship between buoyant density and the age of erythrocytes: Rabbit erythrocytes labeled *in vivo* with a single injection of Fe^{59} Cl_3, 12 μc, were followed in sequential buoyant density analysis.[25] The labeled cells first appear in the lighter fractions. In time these cells become denser (Fig. 10). The width of the Fe^{59} distribution increases with time. Initially about 70 per cent of the cells of given age are found in two adjacent fractions; after 42 days the band width has increased so that only 40 per cent of the labeled cells are in adjacent fractions.

The median of the Fe^{59} distribution progresses linearly with time through the main part of the cell distribution (Fig. 11). This is a necessary consequence in a situation in which a population distribution remains constant while new material is introduced at one end and removed at the other. The agreement between the extrapolated intercept, 61 days, and the reported[26, 27] mean cell life of rabbit erythrocytes, 45–70 days, is another indication that the progression of the label through the distribution corresponds to the aging of the red cells. At late times the progression is observed to stop, presumably because of the reappearance of the label at the light end of the distribution. The nonzero intercept of the mean on the ordinate is attributed to the drainage errors mentioned previously.

The linear progression of the label indicates a direct relation between the average chronological age and buoyant density of erythrocytes. The observed spreading of the labeled cells in the buoyant distribution with time indicates that the erythrocytes "age" at different rates. This is in agreement with the previously observed spread in the survival time of the rabbit cells.[26, 27] In the densest third of the distribution of human erythrocytes, we have observed that the mean cell volume decreases progressively; in the densest fractions the cells are observed to change morphologically, i.e., to become more spherical.

Summary.—A procedure for preparing and fractionating linear density gradients of BSA is described. Buoyant density distributions of human erythrocytes from one individual were reproducible, but significantly different from the distributions of four other normal individuals. The erythrocytes were shown to behave as perfect osmometers. Cells from each density fraction were examined cytologically, and the sizes determined electronically. In aging experiments, Fe^{59} pulse-labeled rabbit erythrocytes entered as light cells and moved linearly with spreading through the density distribution.

It is a pleasure to thank C. M. Pomerat for permission to use the Model B Coulter counter at the Pasadena Foundation for Medical Research, L. Capers and D. Hammond for the cytological studies, and D. Kabat and G. Attardi for permission to cite their unpublished results. This work was supported in part by grant HE 03394 from the USPHS.

* Contribution no. 3045.

[1] Bass, C. G., and F. M. Johns, *Am. J. Trop. Diseases*, **3**, 298 (1915–16).
[2] Ferrebee, J. W., and Q. M. Geiman, *J. Infect. Diseases*, **78**, 173 (1946).
[3] Powell, M. E. A., *Nature*, **193**, 1047 (1962).
[4] Agranoff, B. W., and B. L. Vallee, *Blood*, **9**, 804 (1954).
[5] Kimura, E., T. Suzuki, and Y. Kinoshita, *Nature*, **188**, 1201 (1960).
[6] Borun, E. R., W. G. Figueroa, and S. M. Perry, *J. Clin. Invest.*, **36**, 676 (1957).
[7] Prankerd, T. A. J., *J. Physiol.*, **143**, 325 (1958).
[8] Rigas, D. A., and R. D. Koler, *J. Lab. Clin. Med.*, **58**, 242 (1961).

[9] Garby, L., and M. Hjelm, *Blut*, **9**, 284 (1963).

[10] Borsook, H., J. B. Lingrel, J. L. Scaro, and R. L. Millette, *Nature*, **196**, 346 (1962). These authors fractionated rabbit reticulocytes in BSA density gradients with methods developed in the early stages of this research.

[11] Mateyko, G. M., and M. J. Kopac, *Ann. N. Y. Acad. Sci.*, **105**, 183 (1963).

[12] Bock, R. M., and N. S. Ling, *Anal. Chem.*, **26**, 1543 (1954).

[13] Lakshmanan, T. K., and S. Lieberman, *Arch. Biochem. Biophys.*, **53**, 258 (1954).

[14] Bukantz, S. C., C. R. Rein, and J. F. Kent, *J. Lab. Clin. Med.*, **31**, 394 (1946).

[15] de Duve, C., J. Berthet, and H. Beaufay, *Progr. Biophys. Biophys. Chem.*, **9**, 326 (1959).

[16] Holmquist, W. R., and J. R. Vinograd, *Biochim. et Biophys. Acta*, **69**, 337 (1963).

[17] Borsook, H., E. H. Fischer, and G. Keighley, *J. Biol. Chem.*, **229**, 1059 (1957). The composition of NKM in gm/l is 7.61 NaCl, 0.389 KCl, 1.52 $MgCl_2 \cdot 6H_2O$. The osmolarity is 0.293.

[18] Eagle, H., *J. Exptl. Med.*, **102**, 595 (1955).

[19] Brecher, G., E. F. Jakobek, M. A. Schneiderman, G. Z. Williams, and P. J. Schmidt, *Ann. N.Y. Acad. Sci.*, **99**, 242 (1962).

[20] Kabat, D., and G. Attardi, personal communication.

[21] Ponder, E., *Hemolysis and Related Phenomena* (New York: Grune and Stratton, 1948).

[22] Scatchard, G., A. C. Batchelder, A. Brown, and M. Zosa, *J. Am. Chem. Soc.*, **68**, 2610 (1946).

[23] Hearst, J. E., and J. R. Vinograd, these PROCEEDINGS, **47**, 999 (1961).

[24] This equation may be expressed in terms of tonicity and liquid volume, V. $(d\rho/dV)_{\text{eff}} = d\rho/dV - dT/dV\ d\rho/dT$. The method of evaluating $d\rho/dV$ has been described. The value of dT/dV was obtained as the product $\Delta T/\Delta\rho \cdot d\rho/dV$, where ΔT and $\Delta\rho$ are the difference in tonicity and density, respectively, in the two BSA solutions used in the preparation of the gradient. Buoyant density distributions may be obtained with the above procedures in centrifuge tubes of arbitrary shape.

[25] Clark, P., *Australian J. Sci.*, **22**, 216 (1959).

[26] Burwell, E. L., B. A. Brickley, and C. A. Finch, *Am. J. Physiol.*, **172**, 718 (1953).

[27] Neuberger, A., and J. S. F. Niven, *J. Physiol.*, **112**, 292 (1951).

[28] Farquhar, W. J., and E. H. Ahrens, Jr., *J. Clin. Invest.*, **42**, 675 (1963).

---

*ON THE MANDIBLE OF RAMAPITHECUS**

BY ELWYN L. SIMONS

DEPARTMENT OF GEOLOGY, YALE UNIVERSITY

Communicated by G. E. Hutchinson, December 26, 1963

During the past three years a number of findings have enlarged scientific understanding of the initial differentiation of hominids from pongids. These advances are the outgrowth of significant developments in the study of man-like hominoids of Miocene and Pliocene age, recovered from deposits in Africa and Eurasia. In order of their occurrence these additions to knowledge are as follows: (1) The discovery and description by Dr. L. S. B. Leakey of an African member of *Ramapithecus* [=*Kenyapithecus*] at Fort Ternan, Kenya, in deposits which have been dated by the K/A method as about 14 million years old. (2) The assignment to *Ramapithecus* by Simons[1] of a second maxilla (a referred specimen of *Dryopithecus punjabicus* originally figured by Pilgrim,[2] from Haritalyangar in the Nagri zone, Siwalik Hills, North India). (3) The recent determination at Yale that several known mandibles from the latest Miocene and/or early Pliocene of the Siwaliks can plausibly be referred to *Ramapithecus*. This contribution is an attempt to relate the first two of these discoveries to previously published discussions of